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Effect of amphetamine on extracellular concentrations of amino acids in striatum in neurotensin subtype 1 and 2 receptor null mice: a possible interaction between neurotensin receptors and amino acid systems for study of schizophrenia

Zhimin Li, Yanqi Liang, Mona Boules, Andres Gordillo, and Elliott Richelson

Neuropsychopharmacology Laboratory and Mayo Foundation for Medical Education and Research, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224

Abstract

Neurotensin (NT) is a tridecapeptide that acts as a neuromodulator in the central nervous system mainly through two NT receptors: NTS1 and NTS2. The present study was done to determine the roles of NTS1 and NTS2 on amino acid release in striatum with the use of NTS1 or NTS2 knock-out ($-/-$) mice given *d*-amphetamine. Both NTS1 $-/-$ and NTS2 $-/-$ mice had lower extracellular concentrations of D-serine in striatum than did wild type (WT) mice. NTS2 $-/-$ but not NTS1 $-/-$ mice also had significantly lower basal concentrations of glutamate in striatum as compared to that for WT mice. Systemic administration of *d*-amphetamine (4 mg/kg, ip) increased glutamate release by 500% in WT mice, as compared to 300% in NTS2 $-/-$ mice, and 250% in NTS1 $-/-$ mice. Additionally, *d*-amphetamine injection caused a 4-fold increase in GABA release in both WT and NTS2 $-/-$ mice, but only a 2-fold increase in NTS1 $-/-$ mice. Therefore, NTS1 and NTS2 modulate basal release of D-serine and glutamate, and also *d*-amphetamine-induced GABA and glutamate release in striatum. These results provide further support for the involvement of NT receptors in the pathogenesis of schizophrenia and provide a better understanding of the imbalance of amino acid systems through investigation of a DA-based animal model.

Keywords

neurotensin; schizophrenia; glutamate; D-serine; GABA; amphetamine

Introduction

The dopaminergic system contributes to the pathophysiology of schizophrenia. However, the action of a single neurotransmitter system does not explain all the clinical features of this disease. Thus, studies suggest that schizophrenia results from an imbalance among several interacting neurotransmitter systems including the dopaminergic, serotonergic, glutamatergic, cholinergic, and GABAergic systems (as reviewed in Carlsson et al., 2001). Therefore, the best

Communicating author: Zhimin Li, PhD, Department of Neuropsychopharmacology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, Phone: 904 953 6902; Fax: 904 953 7117; li.zhimin@mayo.edu.

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candidate for treatment of schizophrenia will be a drug that is able to modulate several neurotransmitter systems instead of acting only on the dopaminergic system.

A large amount of evidence gathered over the past two decades suggests that targeting the neurotensin (NT) system may provide a novel and promising treatment for schizophrenia (Breslin et al., 1994; Garver et al., 1991; Sharma et al., 1997). This is because in the central nervous system, NT acts as a neurotransmitter or a neuromodulator affecting several neurotransmitter systems implicated in schizophrenia including dopaminergic, serotonergic, GABAergic, glutamatergic, and cholinergic (Kinkead et al., 1999). Biochemical studies have shown that antipsychotic drug treatments increase NT transmission, and centrally administered NT produces biochemical and behavioral effects similar to those observed following administration of antipsychotic drugs (Binder et al., 2001; Kinkead et al., 1999; Nemeroff 1980).

NT mediates its effects through its receptors: the high affinity NT receptor (NTS1) (Vita et al., 1993), the low affinity NT receptor (NTS2) (Mazella et al., 1996), and NTS3 (Mazella et al., 1998). Both NTS1 and NTS 2 mediate several of the central and peripheral effects of NT and play a major role in the modulation of neurotransmitter systems (Pettibone et al., 2002; Antonelli et al., 2007; Leonetti et al., 2004; Ferraro et al., 2007; Boules et al., 2010). However, very little is known about the physiological role of NTS3.

Several studies show that there are differences in behavior and response to drugs between both NTS1^{-/-} and NTS2^{-/-} mice compared to WT mice : 1) NTS1^{-/-} mice have hyperphagia and abnormal weight gain (Kim et al., 2008); 2) NTS2^{-/-} mice have less freezing response as compared to that for WT mice in a fear-conditioning test (Yamauchi et al., 2007); 3) PD149163, a NT receptor agonist, significantly facilitates prepulse inhibition (PPI) and decreases the acoustic startle response in WT but not NT1 KO mice (Feifel et al., 2010); 4) NT peptide knockout mice (NT^{-/-}) exhibit reduced PPI and no amphetamine-induced disrupted PPI as compared with wide type mice (Kinkead et al., 2005); and 5) Peripheral administration of NT receptor agonists (NT-2 and NT69L) reverses apomorphine-induced climbing in wild type but not in NTS1^{-/-} mice (Mechanic et al., 2009). These data indicate that NT receptors play an important role in animal models related to schizophrenia. The present study was designed to investigate the changes of amino acid levels in the striatum in NTS1 and NTS2 null mice in a DA-based animal model. The results provide support for the idea that both NTS1^{-/-} and NTS2^{-/-} mice provide animal models for studies related to schizophrenia.

Experimental procedure

Animals and housing

NTS1^{-/-}, and NTS2^{-/-} mice were established at Roche (Palo Alto CA, USA) and characterized as described by Mechanic et al., 2009. Wild type C57BL/6J, NTS1^{-/-}, and NTS2^{-/-} mice were used to establish “in house” knockout and wild type colonies through heterozygous mating in each strain. The wild type mice were from the heterozygous progeny. Mice were approximately 2 months of age and weighed 20~25 g at the beginning of the study. All mice were housed in a temperature controlled room (23±2 °C) with a 12:12 light dark cycle (lights on at 0600). Purina 5001 Rodent Chow and tap water were available *ad libitum* at all times. Mice use was approved by Mayo Foundation Institutional Animal Use and Care Committee (IACUC) and was consistent with NIH Guide for the Care and Use of Laboratory Animals.

Surgery

On the day of surgery, mice were anesthetized with gasiform isoflurane (1% isoflurane in a mixture of 20% oxygen and 80% nitrogen gas) and immobilized in a stereotaxic frame (KOPF

Instruments, Tujunga, CA). Anesthesia was maintained during the entire procedure. Each guide cannula (CMA Microdialysis Inc., Acton, MA) was stereotactically implanted into the medial striatum (AP 0.5, L 1.6 V2.5, relative to bregma and skull) (Paxinos and Franklin, 2001), then secured to the skull by screws and dental cement. Following surgery, each mouse was housed individually with food and water *ad libitum* for 3-5 days for recovery from cannulation surgery.

Microdialysis procedure

Microdialysis experiments were carried out on conscious, freely moving mice. On the day of the experiment, the stylet in the guide cannula was replaced with the microdialysis probe (CMA/7 with 2 mm membrane, CMA Microdialysis Inc., Acton, MA). The probe was perfused at 2 μ l/min with artificial cerebrospinal fluid (146 mM NaCl, 1.2 mM CaCl_2 , 3 mM KCl, 1.0 mM MgCl_2 , 1.9 mM Na_2HPO_4 , 0.1 mM NaH_2PO_4 , pH 7.4). After at least 2 h equilibration, dialysate samples were automatically collected every 20 min into vials containing 2 μ l perchloric acid (0.5 M) to delay oxidation of monoamines. Three baseline fractions were collected before saline injection (3 fractions) and then nine samples were collected after *D*-amphetamine injection (4 mg/kg ip). All samples were applied to the capillary electrophoresis with laser induced fluorescence (CE-LIF) detection for the determination of amino acids. Results are reported as % of baseline. The position of the probe was verified by visual inspection at the end of each experiment.

CE-LIF

Amino acids in the microdialysate were measured by CE-LIF (Agilent 3D CE and Picometrics ZETALIF Evolution detector). Briefly, analytes containing primary amine moieties were derivatized by mixing dialysate with a labeling solution [500 mM borate buffer, 43 mM KCN and 5mM naphthalene-2, 3-dicarboxyaldehyde (NDA), pH 8.7] with a reaction time of approximately 15 min in the dark. Before the injection, the capillaries were preconditioned with NaOH 0.5 M, distilled water, and analyze buffer (lithium tetraborate 22.5 mM and lithium dodecyl sulfate 20 mM, pH 9.2) for 3 min each. The samples were injected at a pressure of 0.7 psi for 5 s and the injection volume was approximately 5 nl. After injection, the amino acids were separated in a fused silica capillary of 50 μ m I.D. and 50/65 cm length (effective length/total length) under 18 kv and 20°C. The fluorescence from NDA-labeled analytes was excited with the 442 nm line of a diode laser. Emission was collected on a photomultiplier tube (PMT). D-serine was separated according to (Zhao et al., 2005) with a different buffer (hydroxypropyl- β -cyclodextrin, 40 mM, pH 10.0). The detection limits of glutamate, GABA, D-serine, glycine and aspartate were in nM: 84, 45, 42, 29, and 92, respectively.

Statistical analysis

Two-way repeated-measure ANOVA followed by Tukey test was used to compare the percentage of baseline between groups, time, and treatment as independent factors, and time as the repeated factor using Sigma Stat software (SPSS, Inc., Chicago, IL). Difference in AUC between groups was analyzed by one-way ANOVA using the same software. $P < 0.05$ was considered significant. Student t test was used to compare basal levels of amino acids.

Results

Basal concentration of amino acids

The basal concentrations of D-serine, glycine, glutamate, GABA, and aspartate in striatum are showed in Figure 1. NTS1 and NTS2 null mice had significantly lower basal concentrations of D-serine than that for WT mice ($P = 0.04$). In addition, NTS2^{-/-} mice showed lower basal glutamate levels in the striatum than did WT and NTS1^{-/-} mice ($P = 0.03$). There was no difference in basal levels of glutamate in striatum in WT and NTS1^{-/-} mice ($P > 0.68$). There

was no difference in the basal, striatal concentrations of glycine, GABA, or aspartate between NTS1 null mice, NTS2 null mice and WT mice (All $P > 0.17$).

***d*-Amphetamine-induced changes in amino acid levels in NTS1 and NTS2 null mice**

Systemic administration of 4 mg/kg *d*-amphetamine increased extracellular concentrations of glutamate in the striatum by 5-fold in WT ($P < 0.001$), but only 2- to 3-fold in NTS1 and NTS2 null mice ($P = 0.01$ for NTS2^{-/-}, $P = 0.03$ for NTS1^{-/-}). A significant difference was observed between WT, NTS1 and NTS2 null mice ($P = 0.04$ for NTS2^{-/-}, $P = 0.02$ for NTS1^{-/-}). This observation is supported by a significant Time X group interaction ($P = 0.04$), which is mainly due to a group effect on the time points: 220 mins ($P = 0.03$) and 300 mins ($P = 0.03$). There was no difference in amphetamine-induced glutamate release between the NTS1^{-/-} and NTS2^{-/-} (All $P > 0.37$) (Fig. 2a and 2c).

d-Amphetamine injection increased extracellular concentrations of GABA in the striatum by 400% in WT ($P = 0.006$), 350% in NTS2^{-/-} ($P = 0.009$), and only 100 % in NTS1^{-/-} ($P = 0.04$). Moreover, the *d*-amphetamine-induced increase of GABA release was significantly lower in NTS1^{-/-} mice than in WT and in NTS2^{-/-} mice ($P = 0.01$). This observation is supported by a significant Time X group interaction ($P = 0.04$), which is mainly due to a group effect on the time points: from 140 mins to 300 mins (all $P < 0.05$, except 220 mins) (Fig. 2b and 2c). There was no difference in *d*-amphetamine-induced GABA release between the WT and NTS2^{-/-} mice (All $P > 0.14$).

Discussion

The current study is the first to report the basal concentrations of amino acids in striatum in NTS1 and NTS2 null mice. Our data showed that NTS1 and NTS2 null mice had significantly lower D-serine levels in striatum when compared to WT mice. NTS2^{-/-}, but not NTS1^{-/-}, mice showed lower basal concentrations of glutamate in the striatum. Previous studies have shown that schizophrenic patients have lower levels of D-serine (Hashimoto et al., 2003) and glutamate (Kim et al., 1980) in CSF. D-serine added to antipsychotic drugs for treatment of schizophrenia improves symptoms of schizophrenia (Heresco-Levy et al., 2005). Moreover, our group has shown that the extracellular concentration of DA was higher in the striatum of NTS1 and NTS2 null mice than in WT mice (Liang et al., accompanying manuscript). Taken together, these data suggest that NTS1 and NTS2 null mice may be good animal model for studies related to schizophrenia and that NT receptors are involved in modulating D-serine and glutamate neurotransmissions in the striatum.

Many animal studies have investigated amphetamine-induced glutamate release in the striatum. However, the results are not consistent. Systemic administration of 2.5 mg/kg *d*-amphetamine increases glutamate release by 200% in rat striatum (Gray et al., 1999). Intracerebral infusion of *d*-amphetamine (10 $\mu\text{g}/\mu\text{l}$ and 20 $\mu\text{g}/\mu\text{l}$) also significantly increases extracellular concentration of glutamate in striatum (Del Arco et al., 1999). However, in another report *d*-Amphetamine (2 mg/kg, s.c.) decreases glutamate dialysate concentration over 3 h (Miele et al., 2000). The reasons for these differences are unknown. Nonetheless, our results showed that there is a significant increase of glutamate release following 4 mg/kg *d*-amphetamine injection, thus confirming the work of others (Gray et al., 1999).

It has been proposed that the mechanism of *d*-amphetamine-induced increase of glutamate release involves D₁ receptors, particularly in the ventral tegmental area (VTA), which regulate the excitatory influence of systemic amphetamine on glutamate in the striatum via a trans-synaptic, corticosubcortical loop (Gray et al., 1999). On the other hand, several studies report a functional interaction between DA, the D₂ receptor, and striatal glutamate release (Yamamoto and Davy., 1992). Therefore, amphetamine-induced change of glutamate release is due to a

balance between the D₁ excitatory and D₂ inhibitory modulation (Expósito et al., 1999). Our data show that there was less amphetamine-induced increase of glutamate release in striatum in NTS1 and NTS2 null mice, which may be due to significantly higher D₂/D₁ receptor expression ratio in the striatum of NTS1 and NTS2 null mice as compared with WT mice (as shown in accompanying manuscript). The D₂ inhibitory effect outweighs the D₁ excitatory effect. A previous study has shown that amphetamine-induced exaggerated activation of DA transmission at D₂ receptors mediates the expression of psychotic symptoms in schizophrenia (Lyon et al., 2009). The D₁-D₂-glutamate interaction in striatum might be relevant to both the pathophysiology and the treatment of schizophrenia. Augmentation of D₂ receptor antagonists by D₁ receptor agonists might be useful to promote glutamate transmission in schizophrenia (As reviewed in Laruelle et al., 2003). Additionally, (-)-Stepholidine, a D₁ receptor agonist and D₂ receptor antagonist, induces a pattern of Fos expression in forebrain areas similar to that induced by clozapine (Mo et al., 2005). Therefore, the abnormality of our D₂/D₁ ratio in striatum of NTS1 and NTS2 null mice might contribute to inhibit glutamate-mediated information flow into cortico-striato-thalamic-cortical loops, and might worsen an already deficient glutamate transmission in schizophrenia (Laruelle et al., 2003). Additionally, several studies have suggested that there is an antagonistic NTS₁/D₂ receptor interaction modulating the glutamate release in the striatum (Ferraro et al., 2008, Antonelli et al., 2007). By combining with our results, these data may indicate that NTS1 receptor plays an important role in modulating glutamate transmission, which appears to be deficient in the schizophrenia.

As discussed in the introduction, previous studies have reported behavioral differences relevant to schizophrenia between WT mice and both NTS1^{-/-} and NTS2^{-/-} mice. In studies from our group (Liang et al., accompanying paper), we found that 1) NTS1^{-/-} mice were spontaneously hyperactive and more sensitive to *d*-amphetamine-induced hyperactivity; and 2) NTS1^{-/-} mice had higher basal levels of DA and higher levels of amphetamine-induced DA release in striatum as compared with those for WT mice. Our recent work showed that basal levels of glutamate were significantly lower in the medial prefrontal cortex of NTS1 null mice as compared with those for WT mice and the density of NMDA receptor 2A subunit was reduced in this same area in NTS1^{-/-} as compared to that for WT mice (Li et al., unpublished data).

These observations are consistent with the idea that dysregulation of DA function revealed by amphetamine challenge in schizophrenia stems from a deficit in glutamate transmission (van Berckel et al., 2006). The dysregulation of the DA system in the striatum might further weaken NMDA transmission in corticostriatal glutamate projections (Laruelle et al., 2005). In support of this idea, we showed significant higher D₂/D₁ mRNA ratio in striatum of NTS1- and NTS2-null mice (Liang et al., accompanying paper) in association with apparently deficient glutamate transmission (lower levels of basal and amphetamine-induced glutamate). Noncompetitive NMDA antagonists, such as phencyclidine and ketamine, induce both positive and negative signs and symptoms in healthy subjects and aggravate these signs and symptoms in schizophrenic patients (Krystal et al., 1994; Lahti et al., 1995). Additionally, as mentioned, NMDA agonists including D-serine and glycine significantly improve the symptoms of schizophrenia. Therefore, the lower basal levels of glutamate and D-serine and lower amphetamine-induced glutamate release in the striatum might contribute to the positive and negative schizophrenia-like signs observed in NTS1- and NTS2-null mice.

Our present study shows that systemic administration of *d*-amphetamine increased GABA release in the striatum in all groups, similar to the previous studies that both intrastratial and systemic administration of amphetamine cause a significant increase of the GABA levels in striatum (Del Arco et al., 1998; Porrás and Mora, 1993). However, amphetamine-induced increase of GABA release was significantly lower in NTS1^{-/-} mice than in WT and in NTS2^{-/-} mice. It is proposed that the amphetamine-induced increase of GABA release in striatum occurs when DA and glutamate are released by amphetamine, which may release

GABA by both calcium-dependent and calcium-independent GABA transporter mechanisms (Del Arco et al., 1998). Endogenous DA through both D₁ and D₂ DA receptors plays a role in releasing glutamate and GABA in striatum (Expósito et al., 1999). Our recent data show that there is a relative high D₂/D₁ ratio (around 3) in NTS1^{-/-} as compared with around 1 in WT mice. While the difference between the effect of NTS1 and NTS2 on the amphetamine-induced increase of glutamate and GABA may indicate the different roles of NTS1 and NTS2 in modulation of neurotransmission systems, NTS1 has been reported to play a major role in the modulation of neurotransmitter systems (Pettibone et al., 2002) when compared with NTS2.

In view of these findings and our results, it may be postulated that NT receptors play an important role in the modulation of amphetamine-induced glutamate and GABA neurotransmission in striatum, through a possible interaction with DA receptors (as summarized in Figure 3). NTS1- and NTS2-null mice provide a valuable approach for investigating the treatment and etiology of schizophrenia.

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Figure 1. Basal concentration of amino acids in striatum in NTS1^{-/-}, NTS2^{-/-}, and WT mice. Panel A, Basal concentrations of D-serine, glycine, glutamate, GABA, and aspartate (μM). Panel B, Expansion of the data from Panel A for GABA and aspartate basal concentrations (nM). The basal concentrations were obtained from 3 baseline samples of each animal. All data are shown as mean ± S.E.M (N=5 for each group). *, P<0.05.

Figure 2.

The effect of systemic administration of *d*-amphetamine (4 mg/kg, ip) on amino acid levels in striatum of NTS1^{-/-}, NTS2^{-/-}, and WT mice. Panel A, The effect of amphetamine injection on glutamate release in striatum. The first arrow signifies saline injection (0.9%, NaCl, i.p.) and the second arrow signifies amphetamine injection (4 mg/kg, i.p.). The data are shown as the % of baseline ($\pm$ S.E.M) (N=5 for each group). *, $P < 0.05$, NTS1 and NTS2 null mice versus WT mice. Panel B, The effect of *d*-amphetamine injection on GABA release in striatum. The data are shown as the % of baseline ($\pm$ S.E.M) (N=5 for each group). *, $P < 0.05$ versus WT mice and NTS2^{-/-} mice. Panel C, The effect of *d*-amphetamine injection on both glutamate and

GABA release in striatum. The data are shown as AUC ($\pm$ S.E.M) (N=5 for each group). *, P<0.05.

Figure 3. The diagram incorporates the neurotransmitter interactions and the roles of NTS1, NTS2, D1 and D2 receptors in striatum. +, signifies positive modulation; -, signifies negative modulation.